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Synergistic Effect of Interferon–γ and Mannosylated Liposome–Incorporated Doxorubicin in the Therapy of Experimental Visceral Leishmaniasis

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Active targeting of doxorubicin to macrophages was studied by incorporating it in mannose-coated liposomes by use of visceral leishmaniasis in BALB/c mice as the model macrophage disease. Mannosylated liposomal doxorubicin was more effective than liposomal doxorubicin or free doxorubicin. Because leishmaniasis is accompanied by immunosuppression, immunostimulation by interferon (IFN)–γ was evaluated to act synergistically with mannosylated liposomal doxorubicin therapy. Combination chemotherapy with a suboptimal dose of IFN–γ resulted in possibly complete elimination of spleen parasite burden. Analysis of mRNA levels of infected spleen cells suggested that targeted drug treatment together with IFN–γ, in addition to greatly reducing parasite numbers, resulted in reduced levels of interleukin (IL)–4 but increased levels of IL–12 and inducible nitric oxide synthase. Such combination chemotherapy may provide a promising alternative for the cure of leishmaniasis, with a plausible conversion of antiparasitic T cell response from a Th2 to Th1 pattern indicative of long-term resistance.

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Visceral leishmaniasis is a chronic protozoan infection in humans that is associated with high mortality. It is caused by the protozoan parasite *Leishmania donovani,* which resides and multiplies within macrophages of the reticuloendothelial system [1]. The mainstays of leishmanial therapy are pentavalent antimonials [2]. When treatment fails, second-line agents such as pentamidine and amphotericin B are used [3]. In some cases even these agents fail to eradicate the parasite [4]. Thus, there is a pressing need for a new therapy against this group of diseases.

Doxorubicin, a powerful chemotherapeutic agent used for the treatment of a variety of human cancers, was recently shown to have profound antileishmanial effects [5]. Its therapeutic value, however, is strongly limited by a cumulative dose-dependent cardiotoxicity [6]. A better, alternative approach to circumvent this obstacle is the use of liposome as a carrier for doxorubicin. Various investigators have shown that liposome encapsulation of doxorubicin can increase the therapeutic index by reducing cardiotoxicity, with maintenance or in some cases even improvement of antitumor activity [7]. The natural homing of liposomes by macrophages has been exploited to activate the tumoricidal properties of macrophages by liposomeentrapped immunomodulators [8] and to treat diseases linked to macrophage-resident microorganisms [9] and parasites, including leishmania [10]. A potential approach for promoting the uptake of liposomal content by macrophages is to incorporate ligands capable of interacting with macrophage surface receptors. Taking into account the exclusive presence of mannose receptors on macrophages [11] and the success obtained by delivering drugs by encapsulation in mannose-grafted liposomes [12, 13], we prepared liposomes bearing this sugar on their surface as an alternate means of delivering doxorubicin for the therapy of experimental visceral leishmaniasis.

One major complicating factor in chemotherapeutic treatment is the depressed immune functions exhibited by victims of disseminated leishmaniasis. Appropriate T cell-mediated responses are of primary importance in an effective host defense in visceral leishmaniasis [14]. There is also a correlation between host control over parasite replication and the capacity of T cells to produce interferon (IFN)-γ and interleukin (IL)-2 [15]. Both in humans [16, 17] and in experimental animal models [18, 19], *L. donovani* infection is accompanied by parasite-specific immune depression mediated by T cells and macrophages, thereby preventing spontaneous cure and development of protective immunity. Immunostimulation of the infected host is therefore an effective strategy of circumventing immunosuppression. IFN-γ could be used as an immunopotentiator for stimulation of nonspecific host defense—in particular, for the cells of the mononuclear phagocyte system. Exogenously administered IFN-γ augments the capacity of macrophages to eliminate *Leishmania* infection [20] and acts synergistically with pentavalent antimony [21, 22]. Moreover, the safety of parenteral recombinant human IFN-γ has been demonstrated for various diseases, including leprosy, cancer, and AIDS [23-25].

In this study, we evaluated the effect of doxorubicin encapsulated in mannose-grafted liposomes, alone or in combination with IFN-γ, in the treatment of experimental visceral leishmaniasis. The appeal of the combination chemotherapy stems from its potential to simultaneously attack

intracellular *L. donovani* by different mechanisms: the use of direct toxicity to the protozoan within parasitized macrophages through receptor-mediated targeting of doxorubicin and the use of the activating effects on the macrophage itself.

Materials and Methods

Parasites. *L. donovani* AG83 (MHOM/IN/1983/AG83) was isolated from an Indian patient with kala-azar [26]. The strain was maintained in BALB/c mice (National Institute of Nutrition, Hyderabad, India) by intravenous (iv) passage every 6 weeks. For in vitro culture, promastigotes were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS).

Bone marrow-derived macrophages. Bone marrow-derived macrophages (BMDMs) were prepared, as described by Sayers et al. [27], after marrow was flushed from the femora of 6–8-week old BALB/c mice. Bone marrow cells were suspended in RPMI 1640 supplemented with 100 IU penicillin and 100 μg streptomycin/mL medium, 10% FBS, 5% horse serum, and 10% L929-conditioned media and were incubated at 5% CO_2, 95% humidity, at 37° C. After 5–6 days, when all populations differentiated into macrophages, cells were harvested and used for experiments.

Preparation of doxorubicin-containing liposome (doxosome). Doxorubicin, 11.2 μmol in methanol, was complexed with 5.6 μmol cardiolipin solution in ethanol, and the mixture was evaporated to dryness under N_2. This dried mixture was then added to 28.0 μmol lecithin, 28.0 μmol cholesterol, and 12.0 μmol L-α-phosphatidylethanolamine in chloroform:methanol solution (2:1 vol/vol). The mixture was stirred gently to achieve a homogeneous solution and evaporated to dryness under reduced pressure at 40° C–45° C by a rotary evaporator. Thin, dry lipid films were resuspended in 6 mL of 25 m*M* sodium phosphate buffer, pH 7.2, containing 150 m*M* NaCl. After a 30-min swelling time, the resulting liposomes were stirred with glass beads and sonicated intermittently for 10 min in a probe-type sonicator. Untrapped doxorubicin was separated from liposome-encapsulated drug by ultracentrifugation (model L7-55; Beckman Instruments, Palo Alto, CA) at 105,000 *g* for 90 min (3×). Resuspended liposomal suspension was passed through polycarbonate membrane filters with 0.5-μm pore diameters (Nucleopore; Costar, Cambridge, MA). Aliquots of final liposome suspension were dissolved in ethanol, and the amount of doxorubicin entrapped in liposome was determined by measuring absorbency at 480 nm, according to the method of Mehta et al. [28].

Covalent coupling of p-aminophenyl-α-D-mannopyranoside to doxosome (mandoxosome). Covalent coupling of *p*-aminophenyl-α-D-mannopyranoside to phosphatidylethanolamine of doxorubicin-containing liposome was done according to the method described elsewhere [29]. In short, phosphatidylethanolamine liposome suspension (1 mL) was mixed with 10 mg (dissolved in 2 mL PBS) *p*-aminophenyl-α-D-mannopyranoside. Glutaraldehyde was added slowly to the liposomal suspension, to a 15 m*M* final concentration, and the mixture was incubated for

5 min at 20° C. Uncoupled sugar and glutaraldehyde were removed by dialysis for 24 h against 25 m*M* sodium phosphate buffer, pH 7.2, containing 150 m*M* NaCl. As a negative control, glucose–liposome–doxorubicin (glucose–doxosome) was prepared similarly by coupling *p*–aminophenyl–α–D–glucopyranoside to doxorubicin–containing liposomes. The coupling of sugars on liposomes was monitored in 2 ways [30]: titration of the liposomal phosphatidylethanolamine amino groups with trinitrobenzene sulfonic acid and agglutination of liposomes with concanavalin A. The titration of the liposomal phosphatidylethanolamine amino groups with trinitrobenzene sulfonic acid in the presence of 0.1% Triton X–100 demonstrated that ~80% –85% of the amino groups was modified.

Electron microscopy. Doxosome and mandoxosome were negatively stained with 2% phosphotungstic acid (pH 7.0) and were viewed in a transmission electron microscope (JEM–100 CX; JEOL Ltd., Tokyo) at 60 kV.

Effect of doxorubicin on L. donovani promastigotes. Growth inhibition of promastigotes, at different concentrations of drug, was studied in RPMI 1640 medium. Promastigote densities were estimated by counting samples by use of a hemocytometer (Neubauer; Fisher Scientific, Springfield, MO).

Treatment of parasite–infected macrophages with free doxorubicin, doxosome, and mandoxosome. Macrophages were infected with freshly transformed promastigotes at a macrophage–to–promastigote ratio of 1:10, in RPMI 1640 medium, for 4 h at 37° C. The unphagocytized parasites were removed by washing with medium twice. After 24 h, infected macrophages were incubated with medium containing doxorubicin, doxosome, and mandoxosome, for 3 h at 37° C, at various concentrations. Drugs were then removed by washing, and cells were placed in fresh RPMI medium for an additional 20 h. Cells were then air dried, fixed in methanol, and stained with Giemsa. The numbers of amastigotes in 100–200 macrophages in drug–treated and control cultures were determined. The mean percentage of suppression of leishmania in drug–treated cultures was calculated by considering the number of amastigotes in untreated cultures as 100%.

Treatment of infected mice with drug. The antileishmanial activity of various forms of doxorubicin was tested in BALB/c mice (~20 g body weight) infected with AG83 strain through the tail vein. At 15 days after inoculation of parasites (10^7 parasites/mouse), test drugs (both free and liposome incorporated) in various doses—0.2–mL volume for 4 consecutive days—were injected into the tail vein. Forty–five days after the start of infection, animals were sacrificed, and multiple spleen impression smears were prepared and stained with Giemsa. Spleen parasite burdens, expressed as Leishman Donovan units (LDU), were calculated as the number of amastigotes per 1000 nucleated cells times spleen weight (in grams) [31]. Spleen LDU versus drug dose was plotted; 5 or 6 animals were used for each experimental group.

Protocols for immunostimulation and chemotherapy. Murine recombinant IFN–γ (*Escherichia coli* derived, 1.9×10^7 U/mg protein; Genzyme

Diagnostics, Cambridge, MA) was administered intraperitoneally in mice, at a concentration of 10^4 U per mouse. Two doses of IFN-γ were administered before (days −4 and −2) or after (days +10 and +12) parasite infection, in 2 groups of mice. A third group received 4 doses of IFN-γ (days −4, −2, +10, and +12). Infected mice were treated with various forms of doxorubicin, IFN-γ, or both in combination according to the previously described regimens. A group of untreated mice was used to ascertain an unmodified course of *L. donovani* infection. The parasite burden of control untreated mice 8 days after parasite infection was used as the baseline infection level in subsequent calculation of drug or immunopotentiator efficacy or both. Experiments were terminated after 45 days, and results are expressed as the percent increase or decrease in the spleen parasite burden, compared with the baseline infection level established at day 8 of infection.

Nitrite (NO_2^-) production. Culture supernatants (100 μL) were assayed for NO_2^- by the Griess reagent, according to the method of Ding et al. [32]. In brief, an equal volume of Griess reagent (1% sulfanilamide and 0.1% *N*-1-napthylethylenediamine dihydrochloride in 2.5% H_3PO_4) was incubated with macrophage supernatants for 10 min at room temperature, and absorbency was measured at 550 nm in a spectrophotometer (model 160A; Shimadzu, Tokyo). NO_2^- concentration was determined by use of $NaNO_2^-$ as a standard. Data were expressed as total micromoles of NO_2^- produced by 10^6 cells at the times indicated in Results.

Reverse transcription–polymerase chain reaction analysis. RNA was isolated from spleen cells [33] of normal and infected mice by use of the guanidium isothiocyanate–phenol–chloroform single-step method [34]. First-strand DNA was synthesized at 37° C for 1 h by use of 1 μg RNA in dH_2O (15 μL), 2.5 μL of 5× RT buffer (250 m*M* Tris-HCl [pH 8.3], 375 m*M* KCl, 50 m*M* dithiothreitol, and 15 m*M* $MgCl_2$), 0.5 μL RNase inhibitor (200 U/mL), 1 μL dNTP mix, 0.5 random hexanucleotide primers, and 1 μL Moloney murine leukemia virus reverse transcriptase (200,000 U/mL). Gene-specific primers were designed and used for amplification of the desired cDNA. We added 1 μL sample cDNA solution to 49 μL reaction mixture that contained 5 μL 10× reaction buffer (500 m*M* KCl, 100 m*M* Tris-HCl [pH 8.3], and 1.5 m*M* $MgCl_2$), 1 μL dNTP mix (1.25 m*M* each of dATP, dCTP, dGTP, and dTTP), 0.3 μL (~1.25 U) *Thermus aquaticus* (*Taq*) DNA polymerase, and 32.7 μL sterile water. We added 5 μL of each primer to give a final primer concentration of 250 pmol/mL. The preparations in the microtubes were amplified by use of a 3-temperature PCR system consisting of denaturation at 94° C for 1 min, primer annealing at 55° C for 1 min, and extension at 72° C for 2 min. Product amplified from cDNA could be distinguished from amplified genomic DNA because the primers amplified genomic DNA introns and exons. The number of cycles was determined for samples not reaching the amplification plateau (32 cycles for HPRT; 35 cycles for others) as shown in table 1. The PCR products were end labeled with γ-^{32}P dATP and used as specific oligonucleotide probes. The prepared cDNA was run on 2% agarose gels, transferred onto nylon membranes, and hybridized with the above probes. The membranes were washed and subjected to autoradiography.

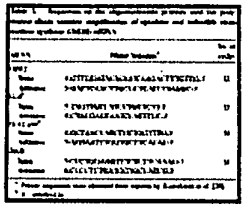

Table 1. Sequences of the oligonucleotide primers used for polymerase chain reaction amplification of cytokine and inducible nitric oxidase synthase (iNOS) mRNA.

Statistical analysis. Results are expressed as mean ± SD of 3 separate experiments with 5 or 6 animals. We used analysis of variance to determine the statistical significance of intergroup comparisons. $P < .05$ was considered to be statistically significant.

Results

Stability of doxosome. Release of doxorubicin from doxosome or mandoxosome in the presence of buffer or plasma was <10% and did not show any marked difference at 4° C or 37° C for 24 h. The addition of plasma had little effect on the rate of drug release. Liposomal integrity was maintained in both doxosome and mandoxosome, as viewed by electron microscopy (figure 1).

Figure 1. Electron micrograph of doxosome *(A)* and mandoxosome *(B)* negatively stained with 2% phosphotungstic acid. Magnification, × 100,000.

Doxorubicin toxicity. All animals given free doxorubicin iv at 20 mg/kg/day for 4 consecutive days died within 10 days of the injection. Animals given free doxorubicin at a dose of 4 mg/kg/day for 4 days survived ≤45 days. However, animals given liposomal doxorubicin at 20 mg/kg/day on the same schedule did not have a higher rate of mortality for ≤45 days. Weight loss, food intake, and other behavioral changes were not studied after drug injection. Discernible toxicity in BMDMs was observed with a doxorubicin dose of 10 μg/mL after 3 h of exposure, as evidenced by gross morphology, trypan blue exclusion, and release of lactate dehydrogenase.

Effect of doxorubicin on L. donovani promastigotes. The effect of doxorubicin on the growth of *L. donovani* promastigotes is shown in figure 2. Doxorubicin was effective against *L. donovani,* and promastigotes could not sustain growth at concentrations >250 ng/mL. To determine whether the drug was killing the organism or inhibiting growth, samples were taken from flasks, placed in fresh medium without doxorubicin, and examined microscopically for growth. Doxorubicin appeared to be a leishmanicidal agent, since it caused a decrease in the number of viable

organisms.

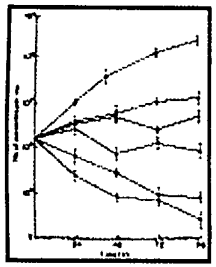

Figure 2. Effect of doxorubicin on growth of *Leishmania donovani* promastigotes. Doxorubicin concentrations (μg/mL): 0 (○), 0.1 (●), 0.25 (△), 0.5 (▲), 1.0 (□), and 10.0 (■).

Treatment of L. donovani–infected macrophages with free doxorubicin, doxosome, and mandoxosome. Inhibition of amastigote multiplication within macrophages by mandoxosome was compared with that by free doxorubicin and doxosome. *Leishmania*–infected cultures were pulsed with various drug forms for 3 h at 37° C in macrophage culture medium. Controls were placed in medium alone. After pulse treatment, cells were washed and placed in drug–free medium for an additional 20 h. They were then stained, and the number of infected macrophages was determined microscopically. Immediately after treatment, it is difficult to differentiate viable from nonviable amastigotes. Therefore, a 20–h interval between drug treatment and staining was chosen to allow for the disposal of dead parasites. Mandoxosome was the most effective of all drug forms tested, with an IC_{50} of 3.4 ng/mL, compared with 480 and 9.6 ng/mL for free doxorubicin and doxosome, respectively (figure 3). Glucose–doxosome was comparable to doxorubicin ($P > .2$). Empty liposome was tested for leishmanicidal effect and was not toxic for intracellular amastigotes. There was no toxic effect on macrophages in vitro by doxosome and mandoxosome at the highest concentration of doxorubicin (100 ng/mL) used as liposomally incorporated drug.

Figure 3. Effects of various forms of doxorubicin (DOX) on growth of *Leishmania donovani* amastigotes in bone marrow –derived macrophages. Infected macrophages were treated with various concentrations of doxorubicin, for 3 h at 37° C, given as free drug (□), doxosome (△), glucose–doxosome (▲), and mandoxosome (○). Infected controls contained 5.85 ± 0.43 amastigotes/macrophage. Data are mean ± SD of 3 experiments.

Combination treatment of infected macrophages, with various forms of doxorubicin and IFN-γ. Unstimulated BMDMs readily supported the replication of *L. donovani,* and 48 h after challenge there was nearly a 2–fold increase in the number of intracellular amastigotes. As shown in figure 3, the addition of various forms of doxorubicin resulted in 50% suppression of parasite growth, at the following concentrations: 480 ng/mL for free

doxorubicin, 9.6 ng/mL for doxosome, and 3.4 ng/mL for mandoxosome. To determine if IFN-γ could act synergistically with doxorubicin, BMDMs were first treated suboptimally with 10 U IFN-γ/mL for 24 h (optimal conditions for inducing BMDM leishmanicidal activity in our laboratory were stimulation with 250 U/mL for 48 h). These cells were infected with *L. donovani* and treated for 3 h with ineffective or low-effective doses of various forms of doxorubicin: 0–2.5 ng/mL, mandoxosome; 0–5 ng/mL, doxosome; and 0–300 ng/mL, free doxorubicin. Under these conditions, the in vitro effect of doxorubicin was appreciably enhanced ($P < .01$). Mandoxosome at 1 ng/mL had a 55% suppressive effect, whereas 3 ng/mL doxosome and 150 ng/mL free doxorubicin had suppressive effects of 51% and 44%, respectively (figure 4). IFN-γ was also effective in augmenting the antileishmanial activity of various forms of doxorubicin, even when added after infection ($P < .05$). Under the latter condition, a higher concentration of IFN-γ (100 U/mL), which by itself had no effect, was required in order to demonstrate this activity. Postinfection treatment with 10 U/mL IFN-γ did not augment the efficacy of doxorubicin.

Figure 4. In vitro antileishmanial activity of various forms of doxorubicin (DOX) in combination with interferon (IFN)-γ. Infected macrophages were treated with various concentrations of doxorubicin for 3 h at 37° C: macrophages pretreated for 24 h with medium alone before infection, ○; macrophages pretreated for 24 h with 10 U IFN-γ/mL before infection, ●; macrophages pretreated for 24 h with medium alone then with 100 U IFN-γ/mL + doxorubicin after infection, △. Data are mean ± SD of 3 experiments.

NO_2^- production by macrophages subjected to combination chemotherapy. Because the effector molecule for the antileishmanial activity of activated macrophages is nitric oxide (NO) [36], we thought it worthwhile to assess the level of NO_2^- (a measure of NO) in macrophages subjected to various combination chemotherapeutic regimens (figure 5*A*). Infected BMDMs produced 0.43 ± 0.03 nmol/10^6 cells, whereas BMDMs activated with 10 U IFN-γ/mL for 24 h produced 3.26 ± 0.31 nmol NO_2^-/10^6 cells. However, preincubation of infected BMDMs with 10 U IFN-γ/mL for 24 h and mandoxosome (1 ng/mL) treatment for 3 h produced 13.02 ± 1.01 nmol NO_2^-/10^6 cells. Similar treatment with free doxorubicin (150 ng/mL) and doxosome (3 ng/mL) resulted in the production of 10.37 ± 1.02 and 11.73 ± 1.03 nmol NO_2^-/10^6 cells, respectively. Moreover, for all drug forms, the NO_2^- release by primed macrophages was increased with an increase in drug concentration (figure 5*B*). In addition to its high leishmanicidal property, doxorubicin acts as a macrophage activator, with a degree of stimulation comparable to IFN treatment [37]. These results, therefore, suggest that increased antileishmanial activity after combination treatment with IFN-γ and doxorubicin may be correlated with increased production of NO.

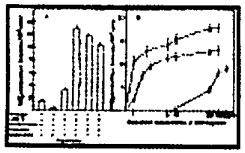

Figure 5. Nitric oxide (NO) production by *Leishmania donovani*–infected bone marrow–derived macrophages (BMDMs). *A*, BMDMs were activated with 10 U interferon (IFN)-γ/mL for 24 h. After wash with culture medium, cells were infected with *L. donovani* and treated after infection for 3 h with suboptimal doses of various forms of doxorubicin: 1, 3, and 150 ng/mL mandoxosome, doxosome, and free doxorubicin, respectively. NO_2^- was measured in culture supernatants after 20 h. *B*, Dose-response curves of various forms of doxorubicin for release of NO_2^- by IFN-γ–primed macrophages. Free drug, □; doxosome, △; mandoxosome, ●. Data are mean ± SD of 3 experiments.

In vivo antileishmanial activity of various drug forms. The efficacy of various forms of doxorubicin for the treatment of visceral leishmaniasis in vivo was determined with a mouse model. BALB/c mice were infected iv with *L. donovani* AG83. The spleen weight increased from 128 ± 23 to 600 ± 34 mg in infected animals 45 days after infection. Animals were given iv injections of free doxorubicin, doxosome, glucose–doxosome, and mandoxosome daily for 4 consecutive days 15 days after infection, and the infection was allowed to proceed for 45 days. Various drug doses were injected at doxorubicin–equivalent concentrations of 0.5–500 μg/kg/day. It is evident from figure 6 that for mandoxosome, 90% of spleen parasites were suppressed at the doxorubicin–equivalent dose of 27.5 μg/kg/day given 15 days after infection. Free doxorubicin at the same dose caused very little (7.5%) reduction in spleen parasite burden, whereas doxosome and glucose–doxosome caused 59% and 62% reduction, respectively, at the same concentration. Mandoxosome was 150× more efficient than free doxorubicin and 5× more efficient than doxosome, on the basis of the estimated dose required for 50% suppression of leishmanial infection.

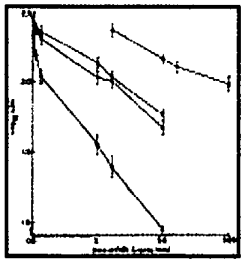

Figure 6. Suppression of spleen parasite burden in murine model of experimental visceral leishmaniasis, by doxorubicin (□), doxosome (△), glucose–doxosome (▲), and mandoxosome (●). Mice were infected with 10^7 *Leishmania donovani* promastigotes, and drugs at indicated doses were administered intravenously daily for 4 consecutive days 15 days after infection. Animals were sacrificed 45 days after infection. Data are mean $\log_{10}$ Leishman Donvan units (LDU) ± SD of 6 animals. $\log_{10}$ LDUs of infected controls was 2.44 ± 0.05.

In vivo antileishmanial effects of combination chemotherapy. To determine whether IFN-γ could also augment the effects of doxorubicin in an in vivo setting, BALB/c mice were subjected to regimens of IFN-γ immunostimulant therapy, various forms of doxorubicin therapy, or a combination of the two. As shown in figure 6, various forms of doxorubicin with 50% antileishmanial activity were selected for the combination therapy: 2, 10, and 300 μg/kg/day for mandoxosome, doxosome, and free doxorubicin, respectively. Three intraperitoneal injections of $>10^5$ U IFN-γ alone, given every other day, halts the visceral replication of *L. donovani* [15]; however, treatment with 2 or 4 injections of 10^4 U produced only

modest inhibition and no killing [15]. Therefore, a dose of 10^4 U IFN-γ was selected to use in combination with the ID_{50} of various forms of doxorubicin. After 45 days of infection, considerable differences were apparent in the parasite burdens of mice, depending on treatment. Untreated mice had an 80-fold increase in infection level above the day 8 baseline infection. Mice treated with IFN-γ alone, after parasite infection (days +10 and +12), did not differ significantly from untreated control mice ($P > .4$). Immunostimulation of BALB/c mice before the parasite challenge (days −4 and −2), however, resulted in parasite burdens at day 45 that were 20% below those of controls ($P < .01$). Treatment with various forms of doxorubicin resulted in a 50% reduction in infection levels, as expected. Combined therapy with IFN-γ and mandoxosome resulted in a synergistic inhibitory effect (figure 7), with a greatly reduced and possibly complete suppression of spleen parasite burden. Indeed, prophylactic or therapeutic treatment with IFN-γ combined with the therapeutic administration of various forms of doxorubicin resulted in *L. donovani* infection that was significantly less than in doxorubicin-treated mice ($P < .001$) and much less than in untreated controls ($P < .0001$). The results also indicate that 4 doses of IFN-γ before and after infection (days −4, −2, +10, and +12) do not amplify antileishmanial effects above those achieved with 2 doses (days −4 and −2; $P > .4$).

Figure 7. Combined effect of interferon (IFN)-γ immunostimulation and doxorubicin therapy in murine model of visceral leishmaniasis. Therapeutic effects are expressed as increase or decrease of spleen parasite burden (Leishman Donvan unit [LDU]), compared with baseline infection level 8 days after parasite challenge. Mice were infected with 10^7 promastigotes, and drugs at respective ID_{50} values were administered daily for 4 consecutive days 15 days after infection. Amastigotes first appeared in spleen on day 8. Infection was allowed to proceed for 45 days. Two groups of mice received IFN-γ intraperitoneally in 2 doses before parasite challenge (days −4 and −2) or after challenge (days +10 and +12). A third group received 4 doses of IFN-γ on days −4, −2, +10, and +12. All data are mean ± SD of 5 mice.

Cytokine production in treated mice. To gain insight into the levels of various cytokines and inducible NO synthase (iNOS) in the spleen after infection with *L. donovani,* we examined the mRNA expression for a Th1 cytokine (e.g., IL-12), a Th2 cytokine (e.g., IL-4), and iNOS, which catalyzes the generation of NO from L-arginine and mediates the leishmanicidal activity of IFN-γ–primed macrophages. Since all three treatment protocols for combination therapy resulted in significant reduction of spleen parasite burden, we selected the therapeutic treatment of IFN-γ (days +10 and +12) along with mandoxosome (days +15, +16, +17, and +18) for the measurement of cytokine mRNAs. RT-PCR analysis of cytokine mRNA levels confirmed that parasite clearance was associated with a dominant Th1 response (figure 8). Thus, *Leishmania*-reactive cells from mice treated with IFN-γ and mandoxosome contained more IL-12 p40 mRNA and less IL-4 mRNA than those from infected untreated controls. Similarly, mice treated with both mandoxosome and IFN-γ also had low levels of parasite-specific IgG1, a Th2-associated isotype, but higher levels of parasite-specific IgG2a, suggesting a more pronounced Th1-type immune response (data not shown). In addition, the iNOS mRNA

expression, which was very low or negligible in the spleen cells of *L. donovani*–infected mice, was considerably induced by the combined therapy of mandoxosome and IFN-γ (figure 8). Taken together, these results indicate that combined therapy with IFN-γ and mandoxosome in mice with established infection potentiates Th1 responses and leads to a significant reduction of spleen parasite burden.

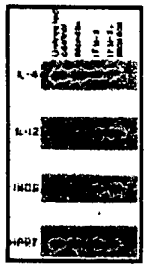

Figure 8. Expression of interleukin (IL)-4, IL-12, inducible nitric oxide synthase (iNOS), and HPRT mRNA by spleen cells of infected mice treated intravenously with various drug regimens and intraperitoneally with interferon (IFN)-γ. Reverse transcription–polymerase chain reaction products were visualized by Southern transfers. RNA samples were obtained from 3 mice in each group. Results are representative of 3 separate samples. HPRT expression levels were used as controls for RNA content and integrity. Mandox, mandoxosome.

Discussion

Recent studies in our laboratory have shown a profound antileishmanial activity for doxorubicin, a widely used anticancer drug [5]. Although used for decades as the treatment of choice for various neoplastic diseases, the anthracycline antibiotic, doxorubicin, has a number of limitations. These include potentially toxic adverse reactions, frequent requirements for high dose or prolonged parenteral administration, and relapse of infection despite apparent cure. For several years, phospholipid vesicles (liposomes) have attracted considerable interest for the selective delivery of drugs and immunomodulators to tissue macrophages [38, 39]. Demonstrable advantages of the targeted delivery vehicle are the relatively slow release of entrapped drug from tissues, reduced quantity of drug required to achieve macrophage cytotoxicity, and augmentation of nonspecific resistance to infection [40]. Furthermore, liposomes are nontoxic and nonimmunogenic [41]. These attractive properties of liposomes prompted an evaluation of the efficacy of doxorubicin entrapped in multilamellar liposomes for treatment of experimental visceral leishmaniasis. The present study demonstrates the potential of a drug-targeting system for specific delivery of active drug moieties to macrophages.

Doxorubicin, a highly cytotoxic and antineoplastic drug, had profound antileishmanial effects when incorporated into mannosylated liposomes. The encapsulated drug was recognized by mannose receptors that are unique to macrophages [11]. The leishmanicidal potency of the drug conjugate has been demonstrated in both an in vitro macrophage model and in a mouse model of visceral leishmaniasis. Although the property of natural homing of liposomes by macrophages has rendered the liposomal doxorubicin much more effective than free doxorubicin, results clearly indicate a substantial increase in the efficacy of the drug incorporated into mannosylated liposomes, compared with that incorporated into nonglycosylated liposomes. Internalization of the mannosylated liposomal drug through the macrophage-mannose receptor has been suggested

because the effect of glucose-liposome-doxorubicin was almost the same as that of nonglycosylated liposome, on the basis ID_{50}. Our group [42] and others [12, 43] have reported additional direct evidence for cell-specific navigation of labeled mannosylated liposomes into the subcellular phagolysome (where *Leishmania* amastigotes are harbored and multiply).

Because of the obvious potential for drug targeting and, thus, increased drug efficacy, mannosylated liposomal doxorubicin was tested in experimental leishmanial infections in mice and in macrophage cultures in vitro. Mannosylated liposomal drug was almost 150× more effective than free doxorubicin in both the in vitro culture and the in vivo animal experiments and was almost 5× more effective than liposomal doxorubicin. During the experimental period, all animals remained healthy, and mannosylated liposomal doxorubicin did not elicit any antibody in mice 4 weeks after treatment, as tested by immunodiffusion.

Because there is no suitable vaccine for visceral leishmaniasis, chemotherapy remains the major method of treatment. However, the drugs commonly used are highly toxic and have severe side effects. Thus, there is a need for additional treatment approaches, such as new chemotherapeutic agents, and for determining whether methods such as nonspecific immunostimulation can act synergistically with liposomal doxorubicin therapy. The results of this study suggest that adjuvant treatment with IFN-γ may be one method by which the efficacy of doxorubicin can be enhanced and/or its dose and presumed toxicity reduced. IFN-γ, a specific and well-characterized immunoregulatory molecule that directly stimulates monocytes and macrophages to act against *L. donovani* [44], is well tolerated at mononuclear phagocyte-activating doses in animals [45] and humans [46]. The superiority of the combined therapeutic regimen suggests that metabolism of doxorubicin or delivery to the parasitized phagolysome may also be favorably influenced by the effects of IFN-γ. Alternatively, despite being unable to kill *L. donovani,* the macrophages treated with low-dose IFN-γ may act, by their reactive nitrogen intermediate antileishmanial mechanisms [36], to render ingested amastigotes more susceptible to doxorubicin action.

Some insight as to how therapy with IFN-γ and mandoxosome influences the production of various cytokines and macrophage NO was gained by examination of mRNA levels in spleen cells shortly after the last day of treatment. Transcript levels of IL-4 were reduced in mice given combined therapy, whereas those for iNOS and IL-12 p40 were significantly elevated. Since IL-4 can suppress both NO and IL-12 p40 production, it is possible that a reduction in the IL-4 level after drug treatment may assist IFN-γ in promoting both macrophage NO and IL-12 production. However, since IFN-γ can prime macrophages to produce IL-12 p40 [47], it is possible that the administration of exogenous IFN-γ, as done in this study, acts as a positive stimulus for enhanced IL-12 production, which in turn may promote both higher IFN-γ production and Th1-cell development. Drug treatment, by reducing numbers of parasites, may also reduce the immunogenic stimulus driving Th2-cell development that predominates during an *L. donovani* infection.

In conclusion, our data show in the treatment of visceral leishmaniasis in mice that a combined regimen of doxorubicin in mannosylated liposomes and IFN-γ is more effective than either treatment alone. Specific formulations of liposomally entrapped doxorubicin are under clinical trial and are in the process of being licensed [48]. The results further indicate that immunostimulation with IFN-γ facilitates leishmanicidal activity of a standard chemotherapeutic drug. The innate toxicity of antileishmanial agents administered in high doses (as often is required to cure visceral leishmaniasis) limits their use. Thus, the need for an effective relatively nontoxic treatment protocol is apparent. Our study provides experimental evidence that immunostimulation combined with an antileishmanial drug is considerably more effective in the treatment of experimental visceral leishmaniasis than either treatment alone. Future validation of the hypothesis tested in our study may encourage trials of this potentially superior therapeutic approach in areas endemic for human leishmaniasis.

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